APPENDIX 1

Practitioner's Docket No. 701039-054701 *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Moses et al	
Application No.:	09/977,878	Group No.: 1642
Filed:	October 15, 2001	Examiner: Canella, Karen
For:	NON-INVASIVE ENZYME SCREEN FOR TISSUE REMODELING ASSOCIATED CONDITIONS	

DECLARATION UNDER 37 CFR 1.131

We, Marsh A. Moses and Li Yan, hereby declare as follows:

1. We are co-inventors of the above-described application.

2. We are aware that the Examiner has cited Monier et al. (Clinica Chimica Acta, Sept 2000, vol. 299: pp 11-23.)

3. Prior to August 17, 2000, we had completed, in the United States, a study using zymography that showed the presence of MMP-9/NGAL migrating at 125 kDa MW in the urine of cancer patients and the absence of this species in non-cancer patients. The 125 kDa MW species was identified as an active MMP-9/NGAL complex using Western Blot and anti-MMP-9 and anti-NGAL antibodies.

4. A copy of notebook pages containing the results of a Zymograph and a Western Blot performed by Dr. Li Yan is attached as Exhibit A, and Exhibit B (2 pages, Western blot and Western Blot data sheet) respectively. Although the dates have been redacted on these notebook pages, we confirm that these pages are dated prior to August 17, 2000. Exhibit A, shows zymogram gels of urine samples from patients with cancer. As indicated by an arrow, a molecular weight species of 125 kDa MW was detected. This species was identified as MMP-9/NGAL complex by Western Blot, see Exhibit B. For the western of Exhibit B, after running a zymogram gel of urine samples from patients with cancer (samples 90, 94, 382, 390 and 442), the proteins were transferred to nitrocellulose and probed with rabbit anti-NGAL antiserum. Anti-NGAL antibody detected the species that runs at 125 kDa MW by zymogram (see arrow, MMP-9-NGAL, Exhibit B). The presence of MMP-9 was confirmed by stripping a blot and probing with anti-MMP-9 antibody. Accordingly, MMP-9/NGAL complex was identified in urine samples from patients with cancer prior to August 17, 2000.

5. Thus, prior to August 17, 2000 we conceived of, in the United States, using a MMP-9/NGAL complex can be used as a diagnostic biomarker for cancer.

6. Accordingly, Moneir et al. teaches no more than what we had already accomplished in the United States prior to its publication date.

7. We diligently continued our laboratory work until the date of filing our patent application.

8. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, and that such willful false statements may jeopardize the validity of the application or any patent that issues therefrom.

2-6-06
Date

Li Yan

2-2-06
Date

Marsha A. Moses

Exhibit A

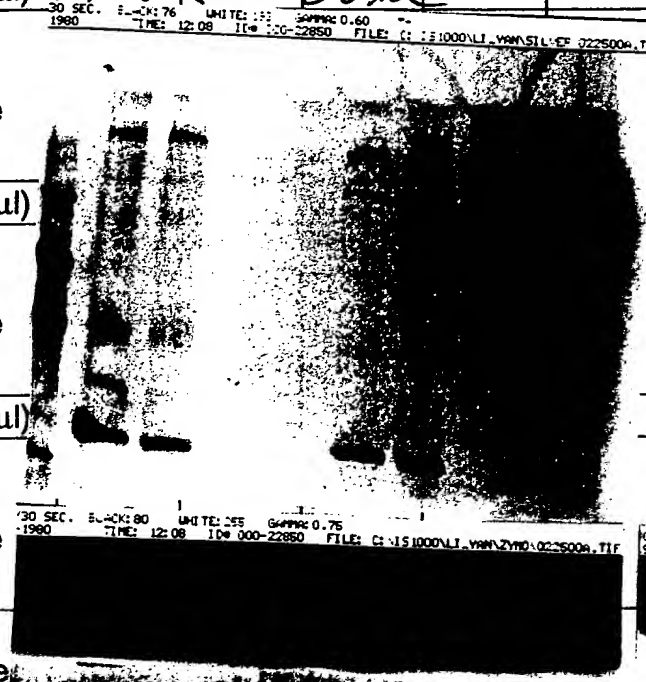
Lab Note 2 – P9 and P10

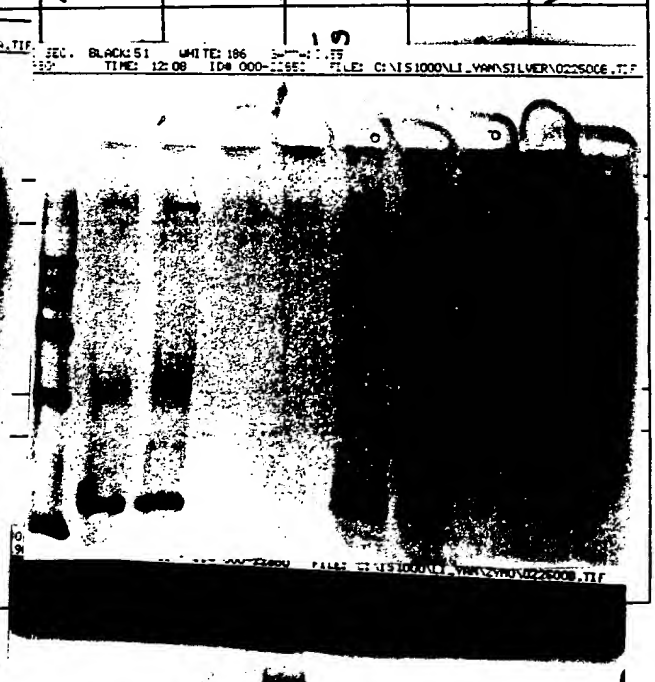
Gelatine Zymograph Data Sheet

Date:

Gel concentration: Bio-Rad %

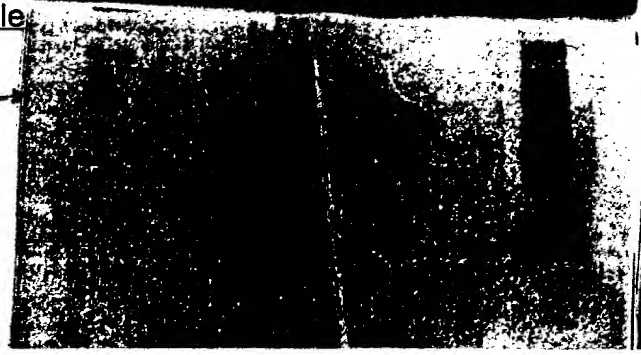
	Lane	1	2	3	4	5	6	7	8	9	10
	Volume (µl)	MW	←	30 µl		→	←	30 µl		→	
10% gelatin	Sample	2.5 µl	Original urine	flow through #144	1% DMSO	10% DMSO	Original urine	flow through #168	1% DMSO	10% DMSO	
	Volume (µl)	MW	←	30 µl		→	←	30 µl		→	
10% gelatin	Sample	2.5 µl	Original urine	flow through #219	1% DMSO	10% DMSO	Original urine	flow through #235	1% DMSO	10% DMSO	
	Volume (µl)	MW	←	30 µl		→	←	30 µl		→	
7.5% SDS PAGE	Sample	2.5 µl	Original urine	flow through #144	1% DMSO	10% DMSO	Original urine	flow through #168	1% DMSO	10% DMSO	
	Volume (µl)	MW	←	30 µl		→	←	30 µl		→	
7.5% SDS PAGE	Sample										
	Volume (µl)										
	Sample										
	Volume (µl)										
	Sample										



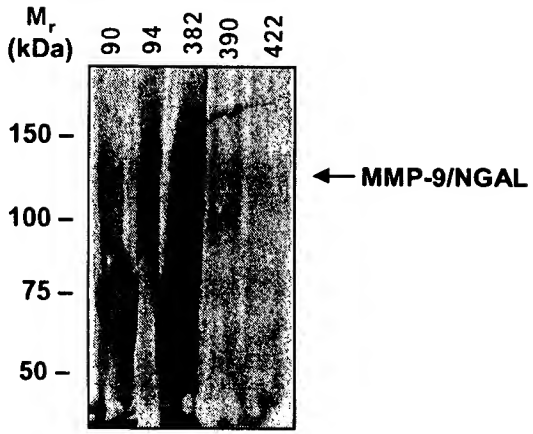


Ele

MMP-9/NGAL →



MOSES LAB



(From the notebook of Dr. Li Yan)

Exhibit B
page 1 of 2

Western Blot Data Sheet

Date:

Gel concentration: 6 % Home-made

Gel loading:

Lane	1	2	3	4	5	6	7	8	9	10
Volume (µl)	~~/~~	5µl	10	10	14.5	14.5	45	28.6	50	~~/~~
			35	35	30.5	30.5	0	16.4	0	
Sample		MW	90 Non-Red	90 Red-	94 Non-Red	94 Red-	382 Non-Red	390 Non-Red	422 Non-Red	

Electrophoresis:

Voltage: 100V Time: 2hrs

Transfer:

Type of filter: 0.2 µm Nitrocellulose Bio-Rad
Methonal: 20%
Voltage: 150V x 40min + 200V + 20min Time: 1 hr

The transfer was pretty complete, except a high mw band was left (~150 kD).

Western blot:

Blocking: 5 % Dry Milk / BSA in TBST / PBS (minutes).
1st antibody:
Antibody name: Rabbit NGAL antiserum
Dilution: 1:1000 in 5 % Dry Milk ~~/ BSA~~
Incubation time: 4°C, ON
Washing: TBST / PBS (minutes) X times
2nd antibo
Ant
Dilu
Inc
Washing:
Developm
Che
Film

	90	94	382	390	422
150+	✓	✓	✓	>Light	—
150	—	—	130+	—	—
125	✓	✓		Light	—
92	✓	✓	✓	✓	—
72	✓	✓	—	✓	—

APPENDIX 2

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Gelatinase isoforms in urine from bladder cancer patients

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Abstract

Matrix metalloproteinases are involved in tumor invasion and metastasis in many types of human carcinomas, in leukocyte infiltration and inflammatory reactions. Three metalloproteinases with gelatinolytic activity were isolated from the urine of patients with untreated high grade bladder cancer or with functioning renal grafts (control). Urinary proteins were fractionated after concentration by continuous-elution SDS–polyacrylamide gel electrophoresis. Collected fractions were analyzed by gelatin zymography and Western blotting. The one-step purification process isolated the gelatinase species from crude urine samples: (1) a 72 kDa progelatinase A (MMP-2) and its actived 68 kDa form; (2) a 92 kDa progelatinase B (MMP-9); (3) a higher molecular weight (HMW) complex (115 kDa) which was identified as progelatinase B associated with lipocalin, NGAL. A similar marker profile was observed in bladder cancer tissues. The current study demonstrated the efficiency of continuous elution electrophoresis. It offered two main advantages: (1) the separation of latent from active gelatinase isoforms with no interference from the TIMPs and (2) the identification and isolation in a single step of large amounts of urine gelatinase species with both high recovery and significant specific activities. Continuous-elution electrophoresis can be used for correlation with clinical events of bladder cancer diagnosis and prognosis.

Keywords: Gelatinase; Neutrophil gelatinase–Associated lipocalin (NGAL); Bladder cancer; Preparative electrophoresis

Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; SDS, sodium dodecyl sulfate; EDTA, ethylene diamine tetraacetic acid

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1. Introduction

Matrix metalloproteinases (MMPs) are important mediators of cancer progression [1, 2]. Overproduction of MMPs by a tumor communicating with the vascular and lymphatic systems might result in increased levels of MMP activity in body fluids such as blood or urine [3,4]. MMPs with gelatinolytic activity were originally identified because they damage type IV collagen basement membrane and have been shown to be critical for tumor cell invasion through matrix proteolysis. Two members of the matrixin family are involved in gelatinolytic activity: gelatinase A (72 kDa, type IV collagenase, MMP-2, EC 3.4.24.24) and gelatinase B (92 kDa, type IV collagenase, MMP-9, EC 3.4.24.35) [5]. As with all MMPs, they are secreted as latent proenzymes which acquire proteolytic capacity after activation through a process involving N-terminal prodomain cleavage [6]. In the body's tissues, gelatinolytic activity is the result of an imbalance between activated enzymes and specific available tissue inhibitors (TIMPs). The main function of TIMPs is to inhibit activated MMPs; but both TIMP-1 and TIMP-2 have been reported to form noncovalent active complexes with progelatinase B and progelatinase A respectively. Research has shown that urine samples from patients with bladder cancer contain either inactive or active gelatinase A and gelatinase B [4]. The purpose of the present study was to validate a convenient and noninvasive procedure for isolating, in a single step, all latent and activated MMPs with gelatinolytic activity in urine, in order to evaluate further new markers of the transition from in situ to invasive bladder carcinoma.

2. Materials and methods

2.1. Patient urine collection and sample preparation

Overnight urine samples (approximately 500 ml to 1000 ml) were collected from patients evaluated in the department of urology and transplantation surgery at the University hospital of Grenoble. After informed consent was obtained, urine samples from patients with functioning renal grafts (control) or with high-grade-stage bladder cancer were collected blindly in sterile bacteriology containers. Prior to analysis, the samples were tested for blood leukocytes and bacteria by using Multistix 8SG (Bayer Diagnostics, Puteaux, France) and contaminated specimens were eliminated. Urinary proteins were pelleted down with 85% (w/v) ammonium sulfate at 4°C. After 20 000 *g* centrifugation for 30 min at 4°C, the pellet was suspended in 50 mmol/l Tris–HCl pH 7.6 containing 50 mmol/l NaCl, and 0.05% (w/v) Brij 35, 0.02% (w/v) NaN_3 (buffer A)

(volume 20–100 ml). The solubilized fraction was dialyzed against the latter buffer and a protease inhibitor cocktail containing 10 μmol/l Nα-p-tosyl-L-lysine chloromethyl-ketone TLCK, 1 mmol/l phenylmethylsulfonyl fluoride PMSF, 1.8 μmol/l leupeptin and 1.5 μmol/l pepstatin (final concentration) was added.

2.2. Gelatinase concentration

Urinary gelatinases were concentrated by incubating the solubilized previous pellet for 1 h and 30 min at 4°C with gelatin–agarose beads (6 mg/ml) [7] equilibrated in 50 mmol/l Tris–HCl pH 7.6 containing 0.5 mol/l NaCl, 5 mmol/l $CaCl_2$, 0.05% (w/v) Brij 35 (buffer B) (one volume of gelatin–agarose per 10 volumes of dialyzed suspension in which 0.45 mol/l NaCl and 5 mmol/l $CaCl_2$ were added). After extensive washing of the beads with buffer B and then with buffer C (50 mmol/l Tris–HCl pH 7.6, 1 mol/l NaCl, 5 mmol/l $CaCl_2$, 0.05% (w/v) Brij 35), the bound material was eluted with 10% (v/v) dimethyl sulfoxide in buffer C. The pooled eluate (12–18 ml) was concentrated 10 times by centrifugation with the centriprep 10 (Amicon, Beverly, MA, USA); 1 ml of the concentrate was mixed with 100 μl of the sample buffer concentrated 10 times (0.63 mol/l Tris–HCl pH 6.7, 0.7 mol/l SDS, 10 mmol/l EDTA and 0.04% (w/v) bromophenol blue, final concentration); it was stored at 4°C until further use.

2.3. Gelatinase isoform fractionation by continuous elution electrophoresis

The experiment was carried out on a Prep Cell Model 491 (Bio-Rad S.A., Munich, Germany) as reported [8]. The latter gelatinase concentrate was loaded onto a 6-cm separative gel of 8% polyacrylamide and a 1.5-cm stacking gel of 4% polyacrylamide which were cast in the 28-mm-diameter gel tube of the instrument. Proteins were electrophoresed vertically at 40 mA for 14 h under cooling conditions, with an anode buffer (25 mmol/l Tris–HCl pH 8.8, which contained 190 mmol/l Glycine, 0.1% SDS (w/v)). Electrophoresis proceeded vertically and individual bands migrated off the bottom of the gel and passed directly into an elution chamber consisting of a thin frit. A dialysis membrane (cut off at 6 kDa) directly underneath the elution frit trapped proteins within the chamber. A peristaltic pump pulled the proteins up through the elution tube with a cathode buffer (25 mmol/l Tris–HCl pH 8.8, which contained 190 mmol/l Glycine, 0.1% SDS (w/v)) at 30 ml/h onto a fraction collector. The above-mentioned protease cocktail inhibitor and 0.05% (w/v) Brij 35 were added to each 3 ml collected fractions which were then analyzed by gelatin zymography.

2.4. Gelatin zymography

Zymography analysis was carried out in 10% (w/v) SDS–polyacrylamide gel under nonreducing conditions and copolymerized with 0.5 mg/ml gelatin, as described previously [7]. The proteins collected from the Prep Cell were applied to the gel in the sample buffer with 2.3% (w/v) SDS but lacking β-mercaptoethanol. The sample was not boiled prior to loading. The gels were washed twice for 30 min in 2.5% (w/v) Triton X-100 in order to remove the SDS. After overnight incubation at 37°C in a 50 mmol/l Tris–HCl, pH 7.6 buffer containing 5 mmol/l $CaCl_2$, 1 μmol/l $ZnCl_2$ and 1% (w/v) Triton X-100, the gels were stained with Coomassie brilliant Blue R-250 and destained as described [9]. Zones of enzymatic activity were shown by negative staining and quantitated by scanning densitometry at 600 nm (CD 60, Desaga, Sarstedt Gruppe). Enzyme activity was expressed in arbitrary units from a standard curve corresponding to the gelatin zymography of increasing concentrations of latent purified gelatinase B [9, 10, 11].

2.5. Immunoblotting

Nitrocellulose film of transferred proteins separated by 10% (w/v) SDS–PAGE was incubated with specific antiserum raised against gelatinase B (1:200), gelatinase A (1:200) or Neutrophil Gelatinase-Associated Lipocalin NGAL (1:1000) [12] followed by the addition of secondary antibody. Protein detection was performed by chemiluminescence (ECL) (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

2.6. Tissue samples

Fresh human urothelial carcinoma tissue samples were obtained from patients who underwent cystectomy or transurethral resection. Immediately after surgical removal the tumor was weighed and proteins were extracted. A tissue homogenate was prepared in 0.1 mol/l Tris–HCl pH 8 by sonication (Branson sonifier, 40 W, 4 X 10s). The medium contained the protease cocktail inhibitor described above. Next, 0.1% (w/v) Triton X-100 was added to the suspension which was homogenized mechanically (Dounce) for a further 10 min at +4°C. The low speed centrifugation supernatant (1000 *g*, 10 min, +4°C) was submitted to a second centrifugation at 100 000 *g* for 60 min at +4°C and the resulting high speed supernatant was stored at −80°C until further use.

2.7. Biochemical assays

Proteins were measured by the Bradford (Bio-Rad Laboratories, CA USA) assay using bovine serum albumin as a protein standard. Differential spectra of

myeloperoxidase were recorded as previously described [13]. Collagenase MMP-8 was measured by ELISA (Amersham Pharmacia Biotech).

3. Results

A preliminary investigation described in Fig. 1 compared the urine of bladder cancer patients with the urine of control patients. After two successive concentration steps (ammonium sulfate precipitation and gelatinase agarose chromatography filtration (Fig. 2)), three gelatinases of 72 kDa, 92 kDa, and 115 kDa molecular weights were recovered from urine samples with a higher expression in bladder cancer urine. The tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2, which are usually present in the urine of healthy subjects and cancer patients, as detected by ELISA, filtrated through the gelatin agarose

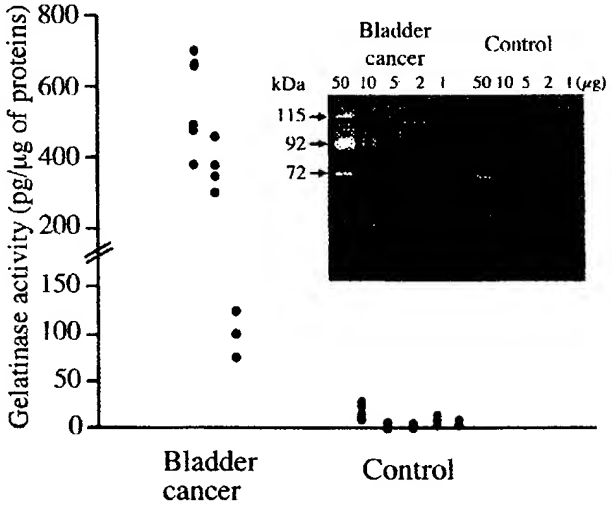

Fig. 1. Gelatinase B activity measurement in urine from bladder cancer patients and controls (functioning renal grafts). Urinary proteins of three patients with bladder cancer and five healthy volunteer subjects were concentrated by $(NH_4)_2SO_4$ precipitation. After solubilization of the pellet, the proteins were determined by Bradford method with albumin as the standard and 50, 10, 5, 2, or 1 µg of proteins were subjected to gelatin zymography. The different results are reported on the diagram; the superposed points correspond to one person (several determinations for the same person). Quantitation was performed by scanning densitometry at 600 nm (CD 60, Desaga, Sarstedt Gruppe); zones of enzymatic activity were shown by negative staining. The results were expressed in pg/µg of proteins compared to a standard curve established from the zymography experiment performed with increasing concentrations of latent purified gelatinase B. *Inset*: one example of gelatin zymography; three metalloproteinases of 72 kDa, 92 kDa and 115 kDa have displayed gelatinolytic activity. Left urinary zymogram for a patient with bladder cancer (several protein quantities were loaded: 50, 10, 5, 2, and 1 µg); Right: control.

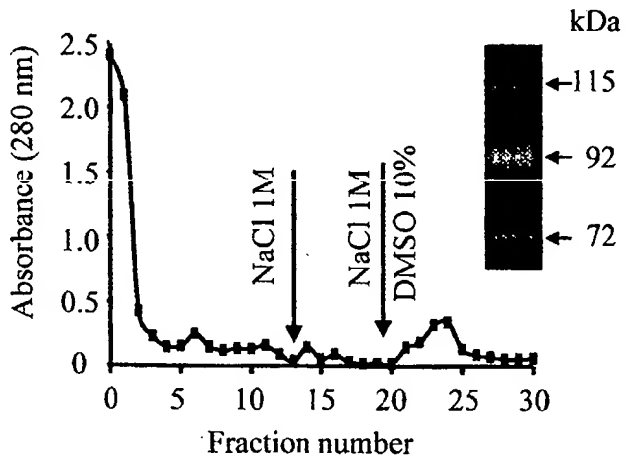

Fig. 2. Affinity binding of urine metalloproteinases with gelatinolytic activity. Urinary proteins were concentrated by $(NH_4)_2SO_4$ precipitation. After solubilization of the pellet they were incubated with gelatin–agarose beads as described in Material and Methods. Metalloproteinases with gelatinolytic activity bound to the matrix and were eluted with 10% DMSO and 1 mol/l NaCl added to equilibrium buffer. *Inset* fractions 21 to 25 (10 ml) were pooled, concentrated onto centricon 10 and submitted to gelatin zymography. Three metalloproteinases of 72 kDa, 92 kDa and 115 kDa have displayed gelatinolytic activity.

matrix [7]; only minute amounts of TIMPs bound to gelatinase and were released during gelatin agarose matrix elution (not shown).

After concentration, the fractionation of the gelatinase pool was carried out through preparative SDS polyacrylamide gel electrophoresis; continuous-elution proceeded under established optimal conditions [8]. The gelatinase elution profile illustrated in Fig. 3A demonstrated the resolution of three activity peaks, whose identity was determined by gelatin zymography (Fig. 3B) and Western blotting (Fig. 3C). Fractions collected at 4 to 5 h 30 min migration time and at 6 to 8 h accounted for the 72 kDa progelatinase and 92 kDa progelatinase B. The third peak of 115 kDa (fractions collected at 9 to 11 h) contained gelatinase B and a 23 kDa polypeptide identified further as Neutrophil Gelatinase Associated Lipocalin (NGAL) by specific polyclonal antibodies (Fig. 3C lane 3) [12]. A similar experiment was carried out with the urine from cancer patients. The panel illustrated in Fig. 4A shows different molecular-weight polypeptides with gelatinolytic activity which was inhibited further by 10 mmol/l EDTA (not shown). Continuous-elution electrophoresis resulted in the fractionation of latent and activated gelatinase isoforms. Pool 1 (fractions collected from 3 to 4 h migration time), pool 2 (fractions from 5 h 48 min to 6 h 48 min), and pool 3 (fractions from 8 h 48 min to 10 h) were submitted to analytical SDS–PAGE (Fig. 4B) confirming that the main polypeptide of pool 1 was 72 kDa gelatinase

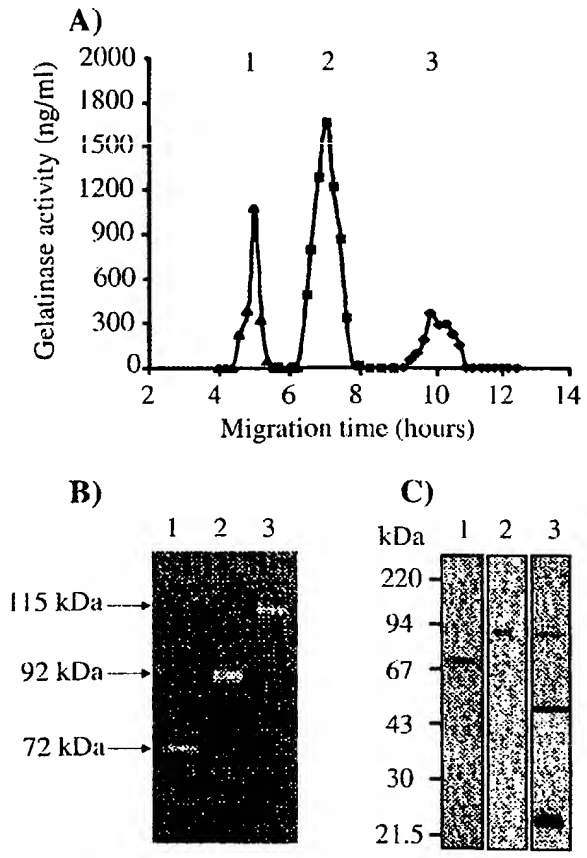

Fig. 3. Continuous-elution electrophoresis of metalloproteinases with gelatinolytic activity recovered from control urine. (A)-One ml of the concentrated protein pool eluted from the gelatin–agarose matrix was loaded vertically on 8% polyacrylamide gel of the Prep Cell instrument for continuous elution through the electrophoresis conditions described in Material and Methods. After elution of the fractionated proteins an aliquot (70 μl) of each fraction (3 ml) was submitted to gelatin zymography. The gelatinase activities (white bands) were quantitated by scanning densitometry at 600 nm (CD60, DESAGA, Starstedt Gruppe). The results were expressed in ng/ml compared to a standard curve established from the zymography experiment performed with increasing concentrations of latent purified gelatinase B. ▲ 72 kDa (1); ■ 92 kDa; (2); ♦ HMW (3) (high molecular weight) (B)-Fractions of peaks 1 (lane 1), 2 (lane 2), and 3 (lane 3), were concentrated 50 to 100 times and pooled onto centriprep 10 (Amicon) for further gelatin zymography. Peak 1: fractions collected between 4 h and 5 h 30 min migration time. Peak 2: fractions collected between 6 and 8 h. Peak 3: fractions collected between 9 and 11 h. (C)-Each peak underwent a 10% SDS–PAGE after β mercaptoethanol denaturation. Western blot experiments were then performed with specific polyclonal antibodies raised against MMP-2 (peak 1, lane 1), MMP-9 (peak 2, lane 2), and MMP-9, NGAL (peak 3, lane 3). Ag/Ab complexes were stained with ECL. The molecular mass standards are represented by kDa.

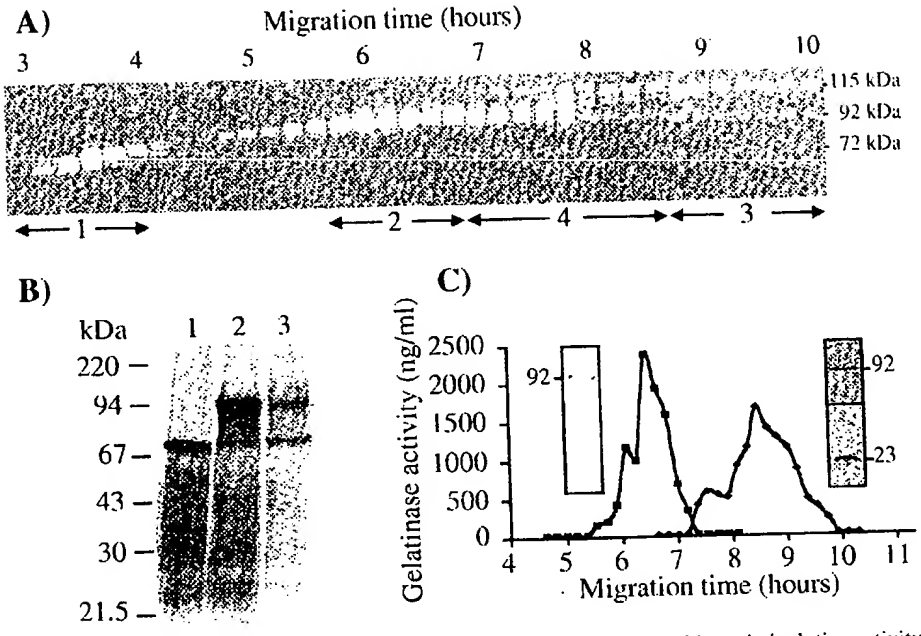

Fig. 4. Continuous-elution electrophoresis of metalloproteinases with gelatinolytic activity recovered from bladder-cancer-patient urine. The process was described in Material and Methods and in Fig. 3. (*A*)–70 μl of each fraction (3 ml) eluted from the Prep Cell instrument were electrophoresed for gelatin zymography. Fractions collected at 3 to 4 h migration time (peak 1), 5 h 48 min to 6 h 48 min migration time (peak 2), and 8 h 48 min to 10 h (peak 3) were pooled and concentrated 70 times onto centriprep 10. Aliquots of each were submitted to 10% SDS–PAGE and silver stained (B). Fractions collected at 7 h to 8 h 36 min migration time (peak 4) were pooled and concentrated to 1 ml. They then underwent a second run of continuous-elution electrophoresis (C). (*B*)–10% SDS–PAGE under reducing conditions and silver stained. *Lanes 1, 2, and 3*: 200 μl of each concentrated peak 1, 2, and 3 respectively were subjected to electrophoresis. The molecular mass standards are represented by kDa. (*C*)-Second run of continuous elution-electrophoresis of peak 4 (fractions collected at 7 h to 8 h 36 min migration time). An aliquot (70 μl) of each fraction (3 ml) was submitted to gelatin zymography. The gelatinase activities (white bands) were quantitated by scanning densitometry as previously done. The results were expressed as ng/ml as described in Fig. 3. *Inset left*: 10% SDS–PAGE under reducing conditions and silver staining of the pooled fractions (5 h 30 min to 7 h migration time). *Inset right*; Western blot experiments of the fractions collected at 8 to 10 h migration time. Ag/Ab complexes were identified with polyclonal antibodies raised against purified MMP-9 [9] and NGAL [11]. Staining was performed with ECL. The 92 is 92 kDa MMP-9, the 23 is 23 kDa NGAL.

(Fig. 4B lane 1) and that of pool 2 was 92 kDa gelatinase (Fig. 4B lane 2), while 3 polypeptide bands were recovered from pool 3 (Fig. 4B lane 3) with molecular weights of 72 kDa, 92 kDa and 105 kDa. Fractions from pool 4 (7 h to 8 h 36 min migration time) were subjected to a second run of continuous-

elution electrophoresis after concentration on centriprep10. Established conditions of electrophoresis removed the 72 kDa polypeptides which had been previously isolated and then resulted in a more resolutive fractionation of the remaining protein mixture, as shown in Fig. 4C. Two peaks were identified: the first eluted peak (fractions collected at 5 h 30 min to 7 h migration time) contained 92 kDa gelatinase as shown by SDS–PAGE (Fig. 4C inset left) while the peak eluted further corresponded to the 92 kDa gelatinase/NGAL complex (Fig. 4C inset right). In order to establish the origin of gelatinases in urine, we examined bladder cancer tissue by zymography (Fig. 5). A 0.1% Triton X-100 soluble extract was shown to contain the three gelatinases 72 kDa gelatinase A, 92 kDa gelatinase B and the 115 kDa molecular weight polypeptide (Fig. 5A, lane 1). The specificity of gelatinolysis was assessed by adding 10 mmol/l EDTA (Fig. 5A, lane 2). It is interesting to note the presence of NGAL (Fig. 5B) which was recently suggested as a marker of primary breast cancer [13]. A possible leukocyte contamination was excluded by MMP-8 and myeloperoxidase measurement which were absent from the sample (not shown) [14] [15].

4. Discussion

This paper is of particular interest for its use of a new continuous-elution procedure of preparative SDS-polyacrylamide gel electrophoresis for the one-step isolation of urine gelatinase isoforms. The urine samples were collected either from patients after renal transplantation with normal renal function (control) or from cancer patients with bladder tumors: they contained both gelatinase A and B types. We and others [16, 17] have demonstrated that various gelatinase species can be identified: indeed according to their latent and (or) activated states, there are four main gelatinase isoforms in urine: free or TIMP-2 and TIMP-1-bound progelatinases, gelatinases with intermediate activation states and fully active enzyme whose prodomain ($\approx$ 10 kDa) has been removed. The latter active gelatinases can be inactivated by either TIMP.

The resolution of continuous polyacrylamide gel electrophoresis was previously demonstrated for protein purification [18, 19]; it has also been used in the isolation of gelatinases from the conditioned medium of human fibrosarcoma HT 1080 cell lines [8]. Efficiency and resolution of preparative SDS–polyacrylamide gel electrophoresis was demonstrated through continuous-elution, which allowed us to highlight several advantages:

1. The total protein sample ($\approx$ 100 μg) recovered after concentration of urine ($\approx$ 1000 ml) can be loaded once onto the polyacrylamide gel.
2. All gelatinase species are isolated in one step and functional properties are preserved.

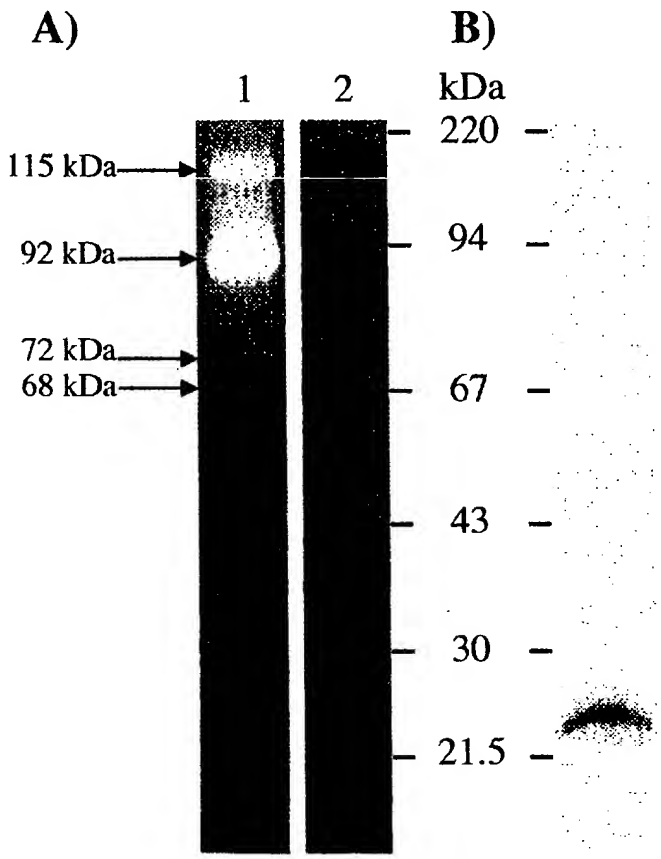

Fig. 5. Gelatin zymography and Western blotting of tumor homogenate from bladder cancer patient. (A)-100 μl of tumor homogenate were added to 10 μl sample buffer 10X and submitted to gelatin zymography. Three metalloproteinases of 72 kDa, 92 kDa, and 115 kDa have displayed gelatinolytic activity; the 68 kDa gelatinase correspond to the active form of gelatinase A (72 kDa) (lane 1). Lane 2: 100 μl of tumor homogenate were added to 10 μl sample buffer 10X and submitted to gelatin zymography; the incubation was carried out in a the presence of 10 mmol/l EDTA. (B)-100 μl of tumor homogenate underwent a 10% SDS–PAGE after β-mercaptoethanol denaturation. The Western blot experiment was then performed with specific polyclonal antibody raised against NGAL. Ag/Ab complexes were stained with ECL. The molecular mass standards were represented by kDa.

3. The one-step purification procedure takes approximately 8 h migration time, giving 80% recovery and highly purified enzymes; for example, the activity of the recovered pooled gelatinase B ranged from 1 to 10 ng/μg of protein and from 30 to 50 ng/μg of protein for control and bladder cancer patients respectively.

4. No TIMP interference was evidenced during the gelatinase measurement as TIMP-1 and TIMP-2 are dissociated from the putative enzyme-inhibitor complexes through SDS–PAGE.

Urine can be stored at −80°C before investigation; however, as a precaution, it is necessary to check that leukocytes and bacteria are lacking. One way to exclude neutrophil contamination and an inflammatory process was to measure myeloperoxidase using differential absorption spectra [14].

NGAL was originally identified as a 23 kDa protein covalently linked to neutrophil gelatinase B in specific granules resulting in a 115–117 kDa NGAL-gelatinase complex [12]. However, the vast majority of NGAL exists independently of gelatinase in myeloperoxidase-negative neutrophil granules either as monomers or heterodimers while gelatinase B is localized on its own in tertiary granules [20, 21]. The function of NGAL is yet unknown. Recently it was associated with neoplastic human tissues, particularly in adenocarcinoma of the lung, colon and pancreas, while renal cell carcinomas contained low NGAL levels [22]. The preliminary results of this study showing NGAL in the urine and tissues of bladder cancer patients merit further investigation. To exclude the possibility that NGAL originates from neutrophil granules, collagenase MMP-8, which is specifically located in specific granules as NGAL, was also investigated (not shown). A lack of MMP-8 in the urine of bladder cancer patients (not shown) suggests NGAL as a putative marker of cancer cells. The tissue inhibitor TIMP-1 has been reported to bind latent and active gelatinase B through C- and N-terminal domains respectively [23]. Moreover, TIMP-1 was recently shown to associate with NGAL [24]. No evidence of a complex formation between TIMP-1 and NGAL as a monomer or a homodimer has been demonstrated in urine.

In bladder cancer there is a clear need for the identification of specific and sensitive markers for the diagnosis and the follow-up of disease progression. This paper focuses on a new resolutive electrophoresis method which specifically identifies the gelatinase isoforms in urine, highlighting activated species. Significantly increased levels of gelatinase A and B and high MW 115 kDa gelatinase B have been detected by zymography in the urine of patients with epithelial cancers including breast, colon and prostate tumors [3]. Bladder washes revealed increased latent gelatinase B but no gelatinase A in patients with evolving bladder cancer compared to controls [25]. Gelatinase increase in the urine of bladder cancer patients has been described by Margulies [4] who found elevated gelatinase A levels. Others have described an increase in gelatinase A in serum or tissue samples of bladder cancer patients [26–28].

The present results obtained by using continuous-elution electrophoresis are promising for a further correlation of activated states of gelatinase with tumor progression. Two subpopulations of human tumor cell lines, Hi-T24 and Lo-T24

were recently investigated to examine the involvement of gelatinases in bladder cancer cells [29]. With the purpose of investigating disease progression, the imbalance between urinary gelatinases and TIMPs and the importance of NGAL are presently under investigation.

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